REMARKS/ARGUMENTS

The amendments set forth above and the following remarks are responsive to the points raised by the Office Action dated January 23, 2008. In view of the amendments set forth above and the following remarks, reconsideration is respectfully requested.

The Pending Claims

Claims 110-111 are added, so that claims 41 and 94-111 are pending.

Claim 41 is amended, and claims 110-111 are added, to describe the invention more clearly. No new matter is added, and support for the amended claim language may be found within the original specification, claims, and drawings. Claims 1 and 110-111 are supported at, for example, paragraphs [0041]-[0042] and [0053] of the specification.

The Office Action

Claims 41, 94-103, 105, 106, 108, and 109 are rejected under 35 U.S.C. § 103 as allegedly unpatentable in view of Hwu et al., *Cancer Res.* 55: 3369-3373 (1995) (hereinafter, "Hwu") and Munz et al., *J. Immunol.* 162: 25-34 (1999) (hereinafter, "Munz").

Claim 104 is rejected under § 103 as allegedly unpatentable over Hwu in view of Munz as applied to claims 41, 94-103, 105, 106, 108, 109, and further in view of U.S. Patent No. 5,844,075 to Kawakami et al. (hereinafter, "Kawakami").

Claim 107 is rejected under § 103 as allegedly unpatentable over Hwu in view of Munz as applied to claims 41, 94-103, 105, 106, 108, 109, and further in view of U.S. Patent No. 6,410,319 to Raubitschek et al. (hereinafter, "Raubitschek").

Each of these rejections is separately and respectfully traversed.

Discussion of the Obviousness Rejection

According to the Office Action, Hwu teaches a method for preparing tumor reactive lymphocytes comprising a) providing murine tumor infiltrating lymphocytes (TIL) transduced with a recombinant retroviral vector (Mov-γ) encoding a chimeric receptor reactive with ovarian adenocarcinoma cells in the presence of IL-2, and b) co-culturing the

transduced TIL cells with syngeneic MC38 colon tumor cells, which results in a large amount of mIFN-γ production. The Office Action concludes that the TIL cells contain an endogenous T-cell receptor reactive with the syngeneic MC38 cells.

The Office Action argues that Munz supplements the teachings of Hwu by establishing that using allogeneic cells as a T cell stimulus is comparable to the syngeneic/autologous stimulation in obtaining potent tumor reactive CTL cells. The Office Action concludes that it would have been obvious to one of ordinary skill in the art to modify the process taught by Hwu with that of Munz by co-culturing either syngenic or allogenic APCs with T cells for activation.

The obviousness rejection cannot be maintained because Hwu and Munz fail to teach each and every element of amended independent claim 1.

Amended independent claim 1 recites "selecting and specifically amplifying lymphocytes comprising an endogenous receptor reactive with a pre-selected antigen capable of inducing proliferation." The lymphocytes comprising the endogenous receptor reactive with the antigen are transduced with a chimeric receptor gene.

Hwu teaches transducing murine TILs with chimeric receptor genes, and then co-culturing the transduced TILs with MC38 tumor cells. Hwu teaches that the TIL cultures produced large amounts of mIFN-γ when co-cultured with the MC38 tumor cells.

Hwu does not teach selecting and specifically amplifying lymphocytes comprising an endogenous receptor reactive with a pre-selected antigen capable of inducing proliferation, as claimed. Any T-cells reactive with the syngeneic MC38 tumor cells in Hwu would not be reactive with an antigen capable of inducing proliferation, as claimed. Tumor antigens are weak self-antigens (See, e.g., Murphy et al. *Cancer Gene Therapy* 14, 499-508 (2007), page 505, 1st full par. left column; Kershaw et al. *Nature Biotechnology* 20, 1221-1227 (2002), page 1221, 1st par., copies attached herewith). Accordingly, tumor antigens would not be strong enough to *select and specifically amplify* lymphocytes comprising an endogenous receptor reactive with the antigen, as claimed.

Moreover, the transduced T-cells that were co-cultured with MC38 tumor cells in Hwu are not reactive with a *pre-selected* antigen. Even if the TIL cultures in Hwu produced

large amounts mIFN-γ when co-cultured with the MC38 tumor cells, as alleged in the Office Action, it is completely unknown in Hwu which MC38 tumor cell antigens are stimulating the TIL cells. Accordingly, such cells cannot be said to be reactive with a *pre-selected* antigen, as claimed. Not only are the tumor antigens in Hwu not strong enough to select and specifically amplify T cells, but Hwu provides no pre-selected antigen with which to immunize a patient to stimulate the T-cells upon adoptive immunotransfer, as is possible with the claimed methods (see par. [0053] of specification).

Moreover, there is no teaching or suggestion in Hwu of selecting and specifically amplifying lymphocytes comprising an endogenous receptor reactive with a pre-selected antigen capable of inducing proliferation, as claimed in amended claim 1. In Hwu, The transduced TILs are simply co-cultured with MC38 tumor cells. There is no teaching or suggestion of selecting and specifically amplifying TILs comprising an endogenous receptor from this coculture. Therefore, it cannot be said that Hwu selects and specifically amplifies, from a mixed population of cells, lymphocytes comprising an endogenous receptor reactive with an antigen capable of inducing proliferation, as claimed in claim 1.

Selecting and specifically amplifying, from a mixed population of cells, lymphocytes comprising an endogenous receptor reactive with a *pre-selected* antigen capable of inducing proliferation has the distinct advantage of making it possible to obtain a higher percentage of active, functional T-cells that are reactive to the pre-selected antigen. Selecting and specifically amplifying the T-cells reactive with a pre-selected antigen capable of inducing proliferation, as claimed, also makes it possible to obtain a purer culture of reactive T-cells and also makes transduction more effective.

Munz does not cure the deficiencies of Hwu. Munz does not teach the concept of selecting and specifically amplifying, from a mixed population of cells, lymphocytes comprising an endogenous receptor reactive with a *pre-selected* antigen capable of inducing proliferation, and transducing the lymphocytes, as claimed in amended claim 1. Accordingly, Hwu and Munz do not teach each and every element of amended claim 1. Therefore, the combination of Hwu and Munz cannot render amended independent claim 1 obvious.

Moreover, the obviousness rejection cannot be maintained because one of ordinary skill in the art reading Hwu and Munz would not have been motivated to select and

specifically amplify T cells having an endogenous receptor reactive to a pre-selected antigen capable of inducing proliferation and transduce the T cells with tumor reactive chimeric TCR genes. In fact, as explained more fully below, Hwu teaches away from the claimed method.

According to the Office Action, one of ordinary skill in the art would have been motivated to modify the teachings of Hwu because of the benefits taught by Munz.

Hwu fails, however, to teach or suggest T cells comprising a tumor reactive chimeric TCR *and* an endogenous TCR reactive with a pre-selected antigen capable of inducing proliferation. In contrast to the presently claimed method, in which alloreactive lymphocytes are *selected* and *specifically amplified*, Hwu teaches that the transduction of genes encoding chimeric TCRs into T cells *eliminates* the need to isolate naturally occurring T cells with a particular antigen specificity, i.e., with a particular native TCR. See, the first three sentences of the Discussion Section. Accordingly, Hwu specifically teaches away from the claimed method of selecting and specifically amplifying lymphocytes with an endogenous receptor reactive with a pre-selected antigen capable of inducing proliferation. In view of the foregoing, one of ordinary skill in the art, after reading Hwu as a whole, would not have been motivated to select and specifically amplify lymphocytes comprising an endogenous receptor reactive with a pre-selected antigen capable of inducing proliferation, and transduce the T cells with tumor reactive chimeric TCR genes, as claimed in amended claim 1.

Munz simply teaches T cells comprising a single tumor reactive, alloreactive TCR. There is nothing in Munz that would motivate one of ordinary skill in the art to select and specifically amplify lymphocytes comprising an endogenous receptor reactive with a pre-selected antigen capable of inducing proliferation, and transduce the T cells with tumor reactive chimeric TCR genes, as claimed in amended claim 1. Therefore, Munz does not cure the deficiency of Hwu.

Amended independent claim 1 is patentable for the reasons set forth above. The dependent claims are also patentable because they depend from patentable amended independent claim 1. The fact that Kawakami may teach a stimulator:responder cell ratio between 3:1 to 10:1, or that Raubitschek may teach a rapid expansion protocol, is of no importance to the patentability of amended independent claim 1. Kawakami and Raubitschek

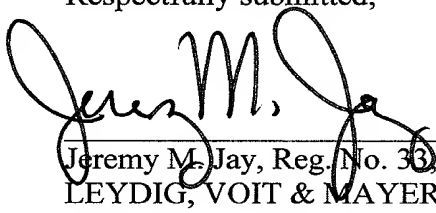
fail to cure the deficiencies of Hwu and Munz, and therefore, the combinations also fail to render the present claims obvious.

New claims 110 and 111 are also patentable, not only because they depend from patentable independent claim 1, but also because they define limitations not taught by the cited references. Neither Hwu nor Munz teaches pre-selecting and specifically amplifying, from a mixed population of cells, lymphocytes comprising an endogenous receptor that is reactive with a pre-selected antigen capable of inducing proliferation, and transducing the pre-selected cells, as claimed in claims 110 and 111. Therefore, claims 110 and 111 are also patentable over the cited references.

Conclusion

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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JMJ/tdh

Cancer Gene Therapy (2007) 14, 499–508
 0929-1903/07 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

Antitumor activity of dual-specific T cells and influenza virus

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Activation and expansion of T cells are important in disease resolution, but tumors do not usually satisfy these immune requirements. Therefore, we employed a novel strategy whereby dual-specific T cells were generated that could respond to both tumor and influenza virus, reasoning that immunization with influenza virus would activate and expand tumor-specific cells, and inhibit tumor growth. Dual-specific T cells were generated by gene modification of influenza virus-specific mouse T cells with a chimeric gene-encoding reactivity against the erbB2 tumor-associated antigen. Dual-specific T cells were demonstrated to respond against both tumor and influenza *in vitro*, and expanded *in vitro* in response to influenza to a much greater degree than in response to tumor cells. Following adoptive transfer and immunization of tumor-bearing mice with influenza virus, dual-specific T cells expanded greatly in numbers in the peritoneal cavity and spleen. This resulted in a significant increase in time of survival of mice. However, tumors were not eradicated, which may have been due to the observed poor penetration of tumor by T cells. This is the first demonstration that the potent immunogenic nature of an infectious agent can be utilized to directly impact on T-cell expansion and activity against tumor *in vivo*.

Cancer Gene Therapy (2007) **14,** 499–508. doi:10.1038/sj.cgt.7701034; published online 2 March 2007

Introduction

T cells of the immune system possess exquisite specificity together with powerful and diverse effector mechanisms, which makes them promising agents to use against malignant disease. Indeed, adoptive transfer of tumor-specific T cells has been demonstrated to mediate antitumor effects in a variety of mouse models.[1–3] Patients too can benefit from adoptive immunotherapy, but responses have been largely limited to a proportion of patients with melanoma.[4]

Adoptively transferred T cells do not persist long term in recipients, and this has been postulated as a reason for their limited antitumor effectiveness.[5,6] Nonmyeloablative conditioning before adoptive transfer has been demonstrated to dramatically increase the persistence of adoptively transferred T cells, and preliminary results suggest that this is associated with an increased frequency of patient responses.[7] However, this form of conditioning induces a concerning immunodeficiency and, while producing an apparent homeostatic expansion of transferred lymphocytes, does not specifically activate them.

Therefore, as an alternate approach, we have investigated the generation of dual-specific T cells as a means of providing lymphocytes with tumor specificity on the one hand, and on the other, the ability to become activated and proliferate in response to a relatively powerful immunogenic stimulus.

We have previously described the generation of dual-specific T cells that were reactive with allogeneic stimulator cells and the ovarian cancer-associated antigen, folate-binding protein (FBP).[8] These dual-specific T cells were demonstrated to respond to both FBP^+ tumor cells and allogeneic splenocytes *in vitro*. They were also able to expand *in vivo* following adoptive transfer and immunization of recipient mice with allogeneic cells. However, optimal expansion required multiple injections of large numbers of allogeneic antigen-presenting cells (3×10^7), which may be difficult to achieve when scaled up to the clinical setting.

Therefore, in this study, we investigated using reactivity against virus as the second specificity, reasoning that some viruses produce a robust immune response following a single injection of a relatively small inoculum that is easily administered. This response can result in activation and extensive proliferation of lymphocytes, and can also lead to activation of the innate immune response, through Toll-like receptor (TLR) involvement, which may often be

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Received 23 May 2006; revised 22 July 2006; accepted 29 August 2006; published online 2 March 2007

lacking in the interaction between the endogenous immune system and tumor. The virus we used was the influenza virus, PR/8 strain (H1N1), since the response against this virus is well characterized in mice.[9]

In this study, we describe the production and characterization of dual-specific T cells with reactivity against influenza virus and the tumor-associated antigen, erbB2. Specificity for erbB2 is provided by transduction of influenza virus-specific T cells with a gene encoding a chimeric receptor made up of extracellular single-chain anti-erbB2 linked to intracellular signaling molecules (Figure 1). The biodistribution and persistence *in vivo* of dual-specific T cells was determined following adoptive transfer, together with their impact on intraperitoneal erbB2-expressing tumors.

This study serves as proof of principle that live virus can be used to directly stimulate tumor-specific T cells *in vivo*, and that this stimulation enables these T cells to expand and mediate antitumor activity, even in a rapidly growing tumor model. These findings may have profound implications for the design of improved immunotherapies for cancer, where a naturally robust response against foreign infectious agents might be deployed against a poorly immunogenic, often ignored, target namely tumor.

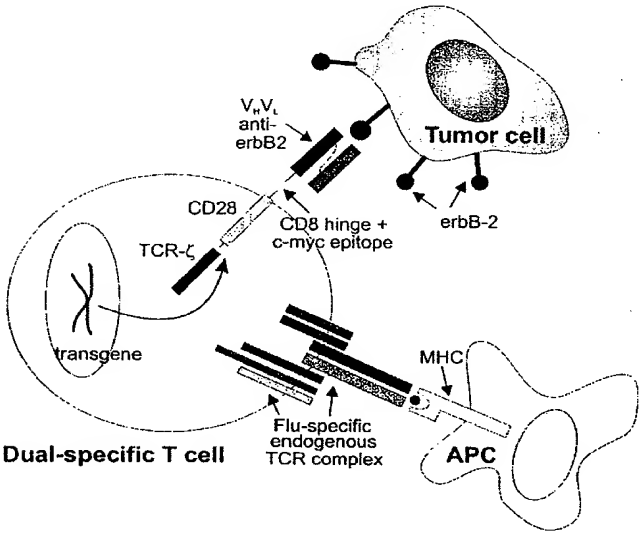

Figure 1 Schematic representation of a dual-specific T cell. Influenza virus-specific T cells are raised in culture using virus-pulsed syngeneic antigen-presenting cells (APC). During their generation, antitumor activity is conferred on them by transduction with a retroviral vector encoding a chimeric anti-erbB2 receptor. The chimeric receptor is made up of extracellular single-chain Fv (V_H and V_L) of anti-erbB2 antibody, linked to transmembrane and intracellular CD28 and then to cytoplasmic domain of the ζ chain of the TCR-CD3 complex. A hinge region of CD8 is included in the recombinant design to enable flexibility in the receptor, and a *c*-myc epitope is included to enable detection of expression by flow cytometry. Therefore, a dual-specific T cell responds via its endogenous TCR against influenza virus processed and presented on MHC molecules by APC, whereas the response against tumor antigen is mediated by the chimeric receptor.

Materials and methods

Cell lines and mice

The mouse breast cancer cell line 4T1.2 was provided by Dr Robin Anderson (Peter MacCallum Cancer Centre (PMCC), Melbourne, VIC, Australia). The erbB2-expressing cell line 4T1.2-erbB2, was generated by transduction of 4T1.2 with a retroviral vector (murine stem cell vector (MSCV)) encoding the cDNA for human erbB2, and was provided by Jacob Jackson (PMCC). The mouse colon cancer cell line CT26 and the mouse kidney cancer cell line Renca were obtained from The American Type Culture Collection (ATCC, Manassas, VA, USA). Derivatives of these cell lines expressing the human tumor-associated antigen, erbB2, CT26-erbB2 and Renca-erbB2, were generated by genetic modification as for 4T1.2-erbB2. Tumor cell lines were maintained at 37°C and 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) medium, supplemented with 10% heat-inactivated fetal calf serum (FCS) (Moregate Biotech, Bulimba, QLD, Australia), 2 mM glutamine (JRH Biosciences, Brooklyn, VIC, Australia), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Sigma, Castle Hill, NSW, Australia).

The murine ecotropic retroviral producer cell lines GP + E86-erbB2 and GP + E86-FBP were generated by transfection of GP + E86 (ATCC) with retroviral expression plasmids containing either pLXSN-scFv-α-erbB2-CD28-ζ,[10] or pSAMEN-scFv-α-FBP-γ[8] as previously described,[11] and were maintained in RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% FCS (Moregate), 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 5×10^{-2} mM 2ME (all from JRH), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). Mouse T cells were cultured in supplemented RPMI, containing 50 IU/ml human recombinant interleukin-2 (rh-IL-2) (Chiron, Emeryville, CA, USA).

BALB/c and SCID mice were purchased from The Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) and housed in specific pathogen-free conditions. Mice of 6–12 weeks of age were used in all experiments, and experiments were performed according to PMCC Animal Experimental Ethics Committee guidelines.

Virus and peptides

Egg-grown A/Puerto Rico/8/34 (H1N1) influenza virus, hereafter referred to as PR/8, was used in the form of infectious allantoic fluid.[12] The infectivity of the virus stock was determined by plaque formation in MDCK cells[12] and expressed as plaque-forming units (pfu). The Class I H-2K^d-restricted epitope NP (147–155), with the sequence TYQRTRALV, from the viral nucleoprotein (NP) is common to all type A influenza virus strains including PR/8 and is immunodominant in BALB/c mice. The Class I H-2K^d-restricted epitope hemagglutinin (HA) (533–541), with the sequence IYSTVASSLI, and Class II I-A^d-restricted epitope HA (126–138), with the sequence HNTNGVTAACSHE are present on the PR/8 HA.

Synthetic peptides representing these epitopes were obtained (Auspep, Parkville, VIC, Australia) and were stored as 10 mg/ml stock dissolved in dimethylsulfoxide (DMSO) (Sigma) at −70°C until use.

T-cell isolation and generation of dual-specific T cells

Two to four weeks before use, BALB/c mice used to make dual-specific T cells were inoculated intranasally with 25–40 pfu each of PR/8 virus to increase the precursor frequency of influenza virus-specific cells.[13] Subsequently, BALB/c mouse splenocytes were isolated by dissecting a spleen, from a PR/8-primed mouse, crushing into 5 ml of hypotonic lysis buffer (pH 7.2–7.4: 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM NaEDTA; Sigma) to deplete red blood cells for 5 min, and straining through a 70 μm filter (Becton Dickinson, San Jose, CA, USA) to produce a single cell suspension. Lysis buffer was diluted by the addition of 5 ml RPMI, and cells were then centrifuged at 1250 r.p.m. (350 *g*) for 7 min, washed once in phosphate-buffered saline (PBS), and the pellets resuspended at 1×10^6 cells/ml in fresh RPMI, containing 1×10^5 pfu/ml PR/8 influenza virus. PR/8 establishes a nonproductive infection in splenic antigen-presenting cells that does not allow generation of new infectious virions, but enables the presentation of viral antigens. Splenocyte/PR/8 suspension (1 ml/well) was added to 24-well plates containing 1 ml overnight cultures of either GP + E86-erbB2 or GP + E86-FBP retroviral producer cell lines (8×10^4 cells/well), and the cells further cultured for 7 days. Recombinant human IL-2 (50 IU/ml) was added after 24 h and then every second day.

On day 7, nonadherent T cells were harvested, and restimulated by coculturing 2×10^5 T cells with 2×10^6 PR/8-pulsed BALB/c irradiated (2000 cGy) splenocytes/well in fresh 24-well plates. On day 9500 μg/ml of a neomycin analog, Geneticin (G418) (Invitrogen, Grand Island, NY, USA) was added to cell cultures. The addition of G418 enables enrichment in culture of transduced lymphocytes by virtue of the neomycin phosphotransferase gene included in the retroviral vector. On days 13–18, transduced cells were harvested and used in experiments. Typically, one BALB/c spleen yielded approximately $0.8–1.4 \times 10^8$ transduced T cells by the end of the culture period.

Cytokine secretion: ELISA

Cytokine secretion in response to influenza virus and tumors was determined by coculturing 5×10^5 dual-specific T cells with either 5×10^5 tumor cells or 5×10^5 BALB/c splenocytes with the addition of either 1×10^5 pfu/ml PR/8 or 1 μg/ml of influenza virus peptide, in 12-well tissue culture plates in a final volume of 1 ml. Following overnight incubation of plates at 37°C/5% CO_2, supernatant was harvested and assayed for IFN-γ in duplicate wells of 96-well flat-bottom plates, using enzyme-linked immunosorbent assays (ELISA), performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

Cytotoxicity assays

The ability of dual-specific T cells to kill tumor cells was assessed by incubating T cells with 2×10^4 ^{51}Cr-labeled target cells (in 200 μl of RPMI) at different effector to target (E:T) ratios, in triplicate wells of a 96-well round-bottomed plate incubated for 4 h at 37°C/5% CO_2. Spontaneous release of ^{51}Cr was determined by incubating the target cells in RPMI alone, and the maximal release was determined by adding sodium dodecyl sulfate (SDS) (Sigma) to target cells, at a 10% final concentration. Following incubation, culture supernatant was harvested using a Skatron Supernatant Collection System (Molecular Devices, Sunnyvale, CA, USA) and radioactivity measured by a Wallac Wizard automatic gamma counter (Amersham, Buckinghamshire, UK).

Proliferation: ^{3}H-thymidine incorporation assays

The ability of dual-specific T cells to proliferate in response to both influenza virus and tumors was assessed by culturing 1×10^5 T cells with either 1×10^5 irradiated tumor cells, or 1×10^5 virus-pulsed, irradiated autologous splenocytes, in triplicate wells of a flat-bottom 96-well plate for 48 h at 37°C/10% CO_2. Splenocytes were antigen pulsed by the addition of PR/8 to the splenocyte media for 2 h at room temperature before irradiation. Tumor cells were irradiated at 16 000–24 000 cGy, whereas splenocytes received 2000 cGy. Tritiated thymidine, 0.5 μCi/well, was added after 24 h of coculture, and cells were harvested 24 h later onto glass fiber filters (Packard, Meriden, CT, USA). Tritiated thymidine incorporation into cellular DNA was measured in a TRICARB 2100TR Liquid Scintilation Counter (Packard).

Tumor growth in mice

Groups of five SCID mice were inoculated intraperitoneally with 2×10^5 Renca or Renca-erbB2 tumor cells. Some groups received 1×10^6 anti-erbB2 T cells or control anti-FBP T cells by intravenous injection 24 h after tumor inoculation. After a further 24 h, some groups received intraperitoneal immunization with 1×10^7 pfu PR/8 influenza virus. Tumor progression was determined by survival of mice, which was defined as 'time until mice became moribund', at which point they were euthanized.

T-cell trafficking

At various time points following CD45.1$^+$ T-cell transfer and immunization of CD45.2$^+$ SCID mice, tissues were taken and analyzed for the presence of CD45.1$^+$ cells by flow cytometry following staining with FITC-conjugated anti-mouse CD45.1 (Pharmingen). Spleens were crushed, and red blood cells lysed using hypotonic buffer. Tumor was dissociated by mincing with scissors and incubation for 2 h at 37°C in DMEM containing hyaluronidase (100 μg/ml), collagenase (1.0 mg/ml) and DNAse (30 U/ml) (Worthington Biochemical, Lakewood, NJ, USA). Peritoneal exudate cells were isolated by rinsing peritoneal cavities with two 5 ml aliquots of PBS and massaging. The amount of CD45.1$^+$ T cells in each tissue was expressed as percent of the total cell number isolated from the tissue.

Results

Dual-specific T cells respond against both tumor cells and influenza virus

Following transduction of influenza virus-reactive T cells with the chimeric anti-erbB2 receptor it was important to determine if they possessed specificity for both the virus and erbB2$^+$ tumor cells. This was initially done by measuring the secretion of IFN-γ by T cells after coculture with various target cells. T cells transduced with either the anti-erbB2 receptor or a control receptor specific for FBP responded against influenza virus-infected syngeneic splenocytes (Figure 2a). Antiinfluenza virus activity was also demonstrated to be polyclonal since T cells secreted IFN-γ in response to several different MHC Classes I and II peptide epitopes of the influenza virus (Figure 2a). The influenza virus-reactive T cells transduced with the anti-erbB2 chimeric receptor were also demonstrated to secrete IFN-γ in response to erbB2$^+$ tumor cells, but not in response to erbB2$^-$ tumor cells (Figure 2b). The requirement for chimeric anti-erbB2 receptor expression for this response was apparent from the lack of activity of control anti-FBP receptor expressing T cells against erbB2$^+$ tumor cells.

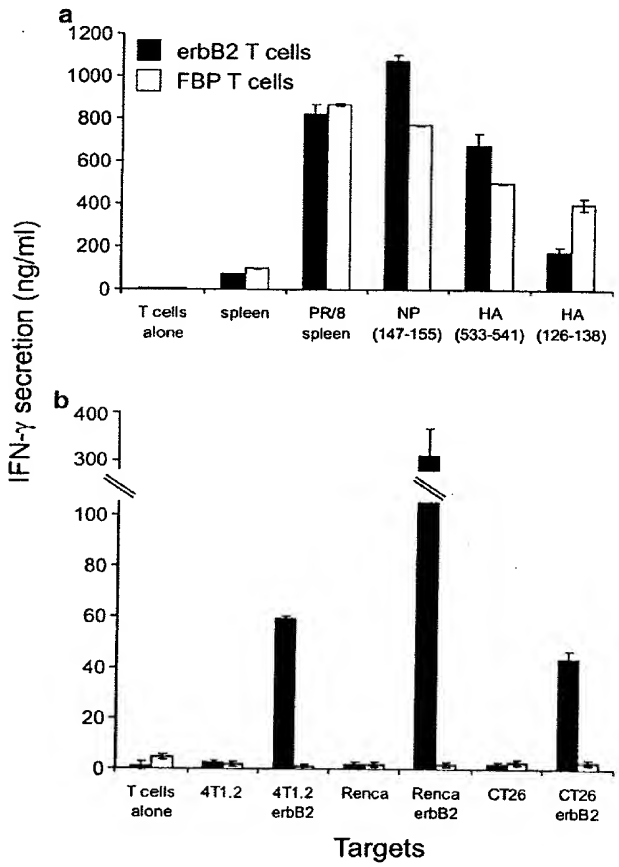

Figure 2 Dual-specific T cells secrete IFN-γ in response to influenza virus and erbB2$^+$ tumor cells. Influenza virus-reactive T cells were transduced with the anti-erbB2 chimeric receptor or the control anti-FBP chimeric receptor, and cultured with either splenocytes pulsed with influenza virus epitopes, or tumor cells with or without erbB2 expression. After overnight incubation, IFN-γ in the supernatant was quantitated using ELISA. **(a)** Both anti-erbB2 and control anti-FBP dual-specific T cells secreted IFN-γ in response to syngeneic splenocytes pulsed with live PR/8 influenza virus (PR/8 spleen) or pulsed with specific influenza virus peptide epitopes (derived from NP, HA) at levels higher than in response to nonpulsed splenocytes (spleen). **(b)** Anti-erbB2-tranduced T cells also secreted IFN-γ specifically in response to erbB2$^+$ and not erbB2$^-$ tumor cells. Anti-FBP-transduced T cells were nonresponsive against erbB2$^+$ tumor cells, thereby demonstrating the requirement for anti-erbB2 transduction for cytokine secretion. This experiment was performed five times with similar results.

Another important T cell function is the ability to kill appropriate target cells, which is particularly important in mediating antitumor effects. We therefore determined the ability of the dual-specific T cell population to lyse erbB2$^+$ tumor cells. Anti-erbB2-transduced T cells lysed a variety of erbB2$^+$ tumor target cells, but not erbB2$^-$ target cells (Figure 3). Lysis was mediated by the anti-erbB2 chimeric receptor as anti-FBP-transduced T cells were ineffective at lysing erbB2$^+$ cells.

Thus, it was evident from these *in vitro* functional assays that anti-erbB2-transduced T cells could respond against erbB2$^+$ tumor cells and against influenza virus-infected targets, demonstrating that the T cell population had dual specificity.

Dual-specific T cells proliferate in response to influenza virus but not in response to tumor cells

Central to our aim of increasing the activity and persistence of tumor-reactive T cells by incorporating influenza virus specificity is the ability of the dual-specific T cell population to expand in response to influenza virus. This was determined using a tritiated thymidine incorporation assay following exposure of T cells to tumor cells or influenza virus-pulsed target cells. Both anti-erbB2-transduced and control anti-FBP-transduced dual-specific T cells proliferated in response to stimulation with influenza virus-pulsed syngeneic splenocytes (Figure 4a). However, the proliferative response of anti-erbB2 dual-specific T cells against tumor cells was low regardless of erbB2 antigen expression (Figure 4a), perhaps indicating that an insufficiently strong proliferative signal was being transmitted through the chimeric receptor following encounter with tumor cells. Nevertheless, the chimeric receptor was capable of transmitting a proliferative signal because anti-erbB2 dual-specific T cells could proliferate following chimeric receptor ligation by plastic-immobilized anti-*c*-myc antibody, which is specific for a 10 amino acid *c*-myc-tag epitope incorporated into the anti-erbB2 chimeric receptor[10] (Figure 4a).

Two potential explanations for the difference between proliferation induced by tumor cells and that induced by anti-*c*-myc antibody were that quantitatively greater numbers of chimeric receptor molecules were triggered through anti-*c*-myc, or that tumor cells secreted an inhibitory factor. To gain insight into this question anti-erbB2 dual-specific T cells were incubated with immobilized anti-*c*-myc in the presence or absence of supernatant

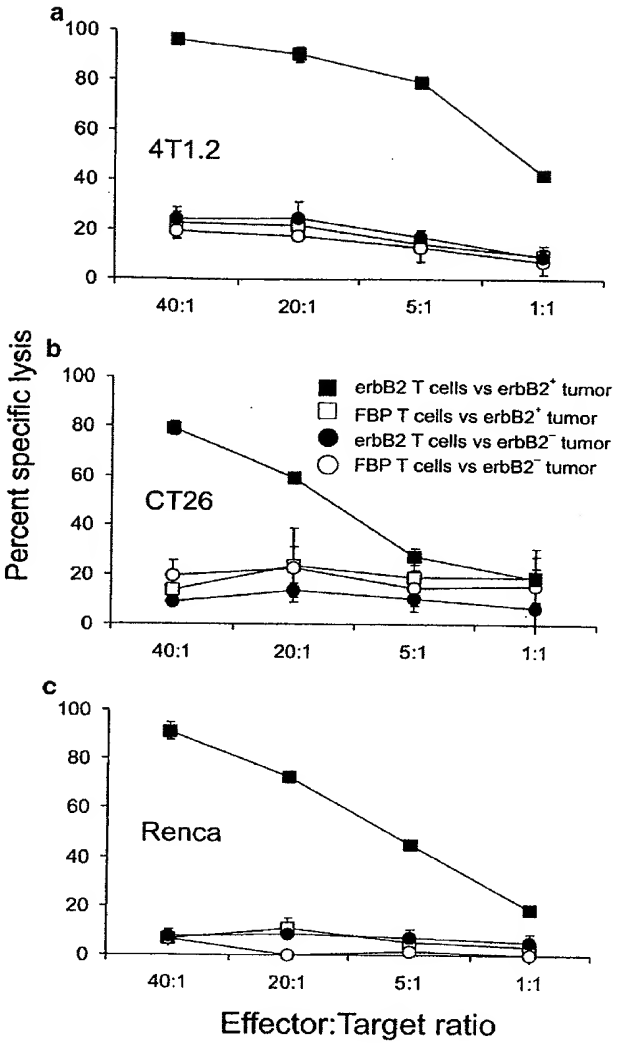

Figure 3 Dual-specific T cells lyse erbB2$^+$ tumor cells. Anti-erbB2-transduced T cells or control anti-FBP-transduced T cells were incubated with ^{51}Cr-labeled erbB2$^+$ or erbB2$^-$ target cells, and percent-specific lysis determined 4 h later. Target cell lines used in the assays were (**a**) 4T1 breast cancer cells, (**b**) CT26 colon cancer cells and (**c**) Renca kidney cancer cells. Although susceptibility to lysis of various erbB2$^+$ cells varied at different effector:target ratios, all three types of erbB2$^+$ cells were lysed by anti-erbB2 T cells, whereas erbB2$^-$ target cells were not lysed. Lysis was dependent on transduction with anti-erbB2 as evidenced by the lack of specific lysis of target cells by anti-FBP-transduced T cells. This experiment was repeated three times with similar results.

taken from actively growing tumor cell cultures. Although T cells proliferated well in response to anti-*c*-myc, the inclusion of tumor cell supernatant in the culture significantly inhibited anti-*c*-myc-induced proliferation (Figure 4b). This suggested that the presence of an inhibitory factor in tumor cell supernatant was responsible for the poor proliferation of dual-specific T cells in response to coculture with tumor cells.

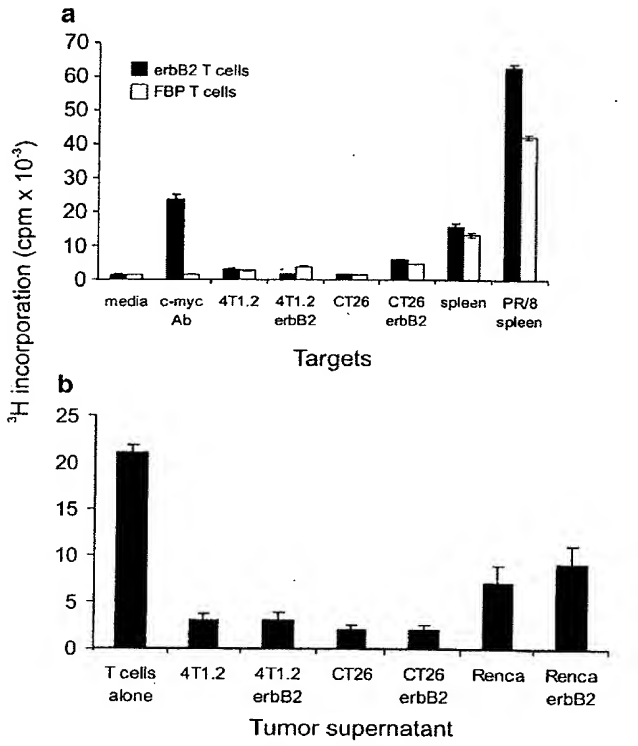

Figure 4 Proliferative capacity of dual-specific T cells in response to influenza virus and tumor. Dual-specific T cells were incubated with the targets listed and proliferation determined after 48 h by measuring tritiated thymidine incorporation. (**a**) Anti-erbB2-, but not anti-FBP-dual-specific T cells proliferated in response to immobilized anti-*c*-myc antibody, but not in response to coculture with irradiated tumor cells expressing erbB2. Syngeneic splenocytes pulsed with the PR/8 influenza virus (PR/8 spleen) could promote proliferation of both anti-erbB2 and anti-FBP T cells to a similar degree ($P_2 = 0.42$). This experiment was performed four times with similar results. (**b**) The presence of 25% (v/v) tumor cell culture supernatant in anti-*c*-myc-stimulated cultures significantly inhibited T cell proliferation ($P_2 < 0.05$, Mann–Whitney test). Values are the mean of four experiments ± s.e.m.

Dual-specific T cells and influenza virus immunization inhibit tumor growth

Having demonstrated the ability of the dual-specific strategy to provide a proliferative signal to tumor-reactive T cells, we next wished to determine if the combination of adoptively transferred dual-specific T cells and immunization with influenza virus could impact on tumor growth *in vivo*.

BALB/c-SCID mice were inoculated intraperitoneal with the BALB/c-derived kidney cancer lines Renca or Renca-erbB2, followed 24 h later with intravenous injection of 1×10^6 dual-specific T cells. After a further 24 h, some mice were immunized intraperitoneal with 1×10^7 live PR/8 influenza virus and mouse survival was determined. Renca-erbB2-bearing mice treated with anti-erbB2 dual-specific T cells alone died over a similar time frame as nontreated mice (Figure 5). However, survival of mice bearing Renca-erbB2 tumors was

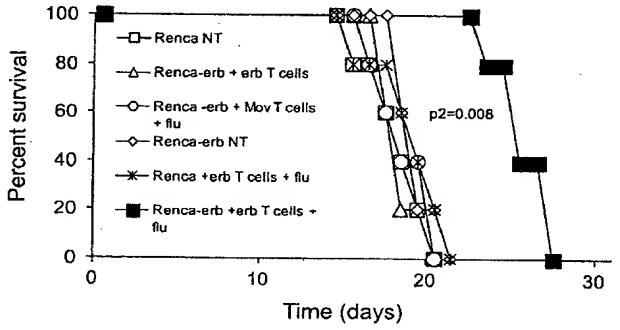

Figure 5 Dual-specific T cells and immunization inhibits tumor growth in mice. BALB/c-SCID mice were injected intraperitoneal with either Renca or Renca-erbB2, followed 1 day later in some mouse groups by intravenous injection of 1×10^6 T cells transduced with either anti-erbB2 or anti-FBP chimeric receptors. Some mice then received intraperitoneal injection of live influenza virus after a further 24 h. Dual-specific T cells alone or influenza virus immunization alone had no impact on mouse survival compared with nontreated (NT) mice. However, mice receiving both anti-erbB2 dual-specific T cells and influenza virus immunization survived significantly longer than all other groups of mice ($P_2 = 0.008$, compared with nontreated, Mann–Whitney test). This experiment was performed twice times with similar results.

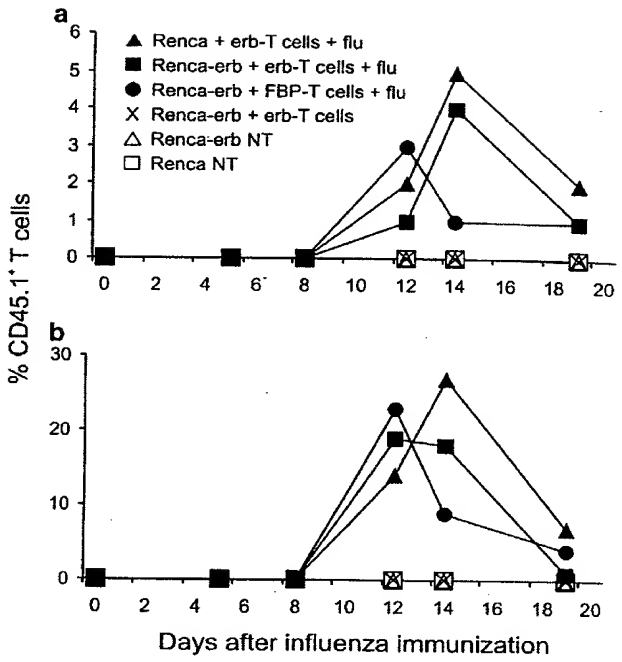

Figure 6 Dual-specific T cells expand *in vivo* in response to influenza virus immunization. CD45.1$^+$ dual-specific T cells were transferred into congenic CD45.2$^+$ mice bearing tumor. Tissues were taken at intervals from groups of three mice, pooled and the percent of tissue comprising CD45.1$^+$ cells determined by flow cytometry. Dual-specific T cells expanded in spleen (**a**) and peritoneum (**b**), in response to influenza virus immunization. The highest number of anti-erbB2 dual-specific T cells was observed between days 12 and 14 following immunization in mice bearing Renca-erbB2 or Renca tumors. Similarly, anti-FBP dual-specific T cells expanded in mice bearing Renca-erbB2 tumors, suggesting expansion was due to the response against influenza virus, and the chimeric receptor played no role in proliferation. Immunization was required for T cell expansion, since no T cells were detected in nonimmunized T cell recipient mice. This experiment was performed twice with similar results.

significantly enhanced by the combination of dual-specific T cells and influenza virus immunization. The antitumor effect was dependent on transduction of T cells with the anti-erbB2 receptor because anti-FBP-transduced T cells and influenza virus immunization did not impact on tumor growth. Similarly, the effect was specific for the erbB2 antigen as growth of Renca tumors lacking erbB2 expression were not affected by transfer of anti-erbB2 T cells and immunization with influenza virus.

Dual-specific T cells expand in vivo *in response to influenza virus immunization*

Although survival of tumor-bearing mice was significantly enhanced by the combination of dual-specific T cells and immunization, tumors were not eradicated, and all mice eventually succumbed to malignant disease. To gain more insight into possible limitations of the therapy, we determined the effect of influenza virus immunization on the expansion and persistence of adoptively transferred dual-specific T cells.

Tumor-bearing mice were injected intravenously with CD45.1$^+$ dual-specific T cells followed 24 h later by intraperitoneal immunization with influenza virus. Intraperitoneal washes and spleens were taken at intervals, and the percentage of dual-specific T cells determined by flow cytometry after staining with anti-CD45.1 monoclonal antibody. T cells first became detectable in large numbers in both intraperitoneal washes and spleen by approximately day 12 after administration of influenza virus, which then decreased to relatively low numbers by day 20 (Figures 6a and b). The presence or absence of erbB2 antigen on tumor cells, or chimeric receptor on T cells did not impact on the extent or kinetics of T-cell expansion, because anti-erbB2 dual-specific T cells expanded in mice bearing either erbB2$^+$ or erbB2$^-$ tumors, and anti-FBP dual-specific T cells expanded in tumor-bearing mice. Immunization with influenza virus was essential for expansion of dual-specific T cells, as they were undetectable in mice receiving dual-specific T cells but no immunization. Therefore, dual-specific T cells expanded *in vivo* to substantial numbers similar in quantity to those associated with disease resolution in other systems.[9] Thus, it seemed unlikely that insufficient numbers of cells alone was responsible for the limited antitumor effect.

Dual-specific T cells do not accumulate in large numbers in tumor

Having demonstrated substantial expansion of T cells in the spleen and intraperitoneal cavity of recipient mice, we next determined the extent of accumulation of T cells in tumor. Reasoning that maximum infiltration of tumor would likely occur when T cell numbers peaked in other

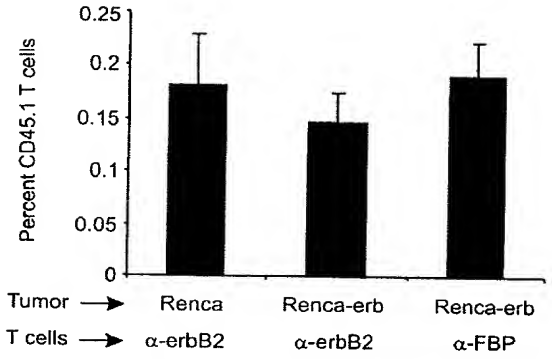

Figure 7 T cells do not accumulate extensively in tumor. CD45.1$^+$ dual-specific T cells were transferred into congenic CD45.2$^+$ tumor-bearing mice and tumors taken 12 days later for analysis using flow cytometry. The percentage of T cells in tumor was low (<1%), and was independent of the type of tumor and T cell. This experiment was performed twice with similar results.

sites, we removed tumors from various groups of mice between days 12 and 14 and determined the percentage of CD45.1$^+$ T cells present using flow cytometry of dissociated tumor following staining with anti-CD45.1 monoclonal antibody. Surprisingly, T cells made up only a very low percentage of tumor tissue (Figure 7), and this did not vary significantly between groups of mice bearing either erbB2$^+$ or erbB2$^-$ tumors, or between groups receiving either anti-erbB2- or anti-FBP-T cells. The percent of T-cell infiltration was below that usually associated with effective responses against tumors or infection in other models.[1,14]

Discussion

Immunotherapy is a promising treatment option for cancer, and significant advances have been made using monoclonal antibodies against lymphoma,[15,16] and cytokines against melanoma, renal cell carcinoma, leukemia and other malignancies.[17] A range of tumor antigens has also been targeted using vaccines or adoptively transferred T cells.[18] However, with the exception of melanoma, tumors have proved to be poorly immunogenic thus far. Indeed, T cells reactive with most common malignancies have been difficult to isolate.

In attempts to generate tumor reactive T cells, genetic modification techniques have been employed resulting in the generation of T cells reactive with ovarian, colon, kidney, breast and other cancers.[19–26] Despite demonstrations of tumor reactivity of genetically redirected T cells, they do not expand or persist *in vivo* even in the continued presence of antigen. In this respect, these previously described gene-modified T cells can be considered to be poorly responsive to antigen. This may be due to the limited signaling capacity of the chimeric genes in comparison with the entire TCR-CD3 complex. Alternatively, it may be due to the non-MHC-restricted nature of antigen recognition that is often a feature of gene-redirected T cells, which results in T-cell encounter with antigen in the absence of optimal costimulation normally achieved in an MHC-restricted interaction with professional antigen-presenting cells.

To overcome the poor immunogenicity of tumor antigens, we and others have generated human dual-specific T cells that could respond to both tumor antigen and a strong immunogen. Immunogens used to date are alloantigen[8] in mouse cells, and Epstein–Barr (virus) EBV[27] and cytomegalovirus (CMV)[28] and influenza virus[29] in human cells. Importantly, the potential utility of dual-specific T cells was highlighted by the demonstration of tumor inhibition in mice using mouse dual-specific T cells recognizing alloantigen and the ovarian cancer-associated antigen FBP.[8] However, a potential limitation to clinical translation using this previously described system was the large doses of allogeneic cells required as immunogen. To circumvent this problem, we investigated the use of another potent immunogen as the second specificity for T cells, namely live virus. The use of live virus to stimulate dual-specific T cells in tumor-bearing mice has not been described before.

In this study, we generated dual-specific T cells that could respond to both influenza virus and the TAA erbB2. Large amounts of IFN-γ were produced in response to influenza virus-pulsed splenocytes, with greater than 800 ng/ml being secreted. Reactivity against influenza virus was also demonstrated to be polyclonal since the T cell population responded to three different influenza virus-derived peptide epitopes. Reactivity was also demonstrated against a range of erbB2$^+$ tumor cells *in vitro*. The levels of IFN-γ secreted in response to individual tumor types varied, with the response against Renca-erbB2 generally higher ($P_2 = 0.002$). This did not appear to be because of differences in the level of erbB2 antigen expression as this was similar between each cell line (data not shown). Potential reasons for the different levels of IFN-γ secretion include differences in expression of adhesion molecules or differential secretion of immune modulators such as TGF-β from some tumor cell lines.

Antitumor activity (40–400 ng/ml IFN-γ) was appreciably less than antiviral reactivity. Nevertheless, considerable amounts of IFN-γ were secreted against tumor cells, and dual-specific T cells were able to specifically lyse a range of tumor cells. The reason for the relatively lower activity of T cells against tumor is not clear, but may be due to a lower signaling capacity of the chimeric receptor compared with the endogenous TCR specific for influenza virus. It would be of interest to determine if modifications to this particular chimeric receptor composition could result in enhanced activity. For example, changes to the hinge region may result in improved function as has been demonstrated previously for some chimeric receptors.[30–32]

It is not clear what proportion of the bulk T cell population were dual specific at a single cell level, although all T cells had integrated the retroviral gene encoding the chimeric receptor and neomycin phosphotransferase, since the neomycin analog G418 was used in the cell culture period. Some insight into the likely

proportion of dual-specific T cells can be gained from previous studies that characterized human dual-specific T cells, which are readily cloned (in contrast to mouse T cells), where 30–40% of T cells in the bulk population were demonstrated to be reactive with two antigens following transduction with a chimeric receptor.[8]

The most striking difference in T-cell response to influenza virus compared with that against tumor was seen in proliferation. T cells could proliferate in response to influenza virus but not in response to tumor cells. Potential reasons for the difference in proliferation included different levels of influenza virus antigen expression compared with TAA expression, inherent differences in signaling ability of TCR compared with chimeric receptor (despite the use of the murine CD28 endodomain), secretion of inhibitory factors by tumor cells or a combination of these reasons. However, the chimeric receptor was determined to be capable of transmitting a proliferative signal as seen by proliferation in response to chimeric receptor ligation by immobilized anti-*c*-myc tag antibody. A contribution from tumor-derived inhibitory factors was supported by assays of T-cell function that included some tumor cell culture supernatant, which inhibited chimeric receptor-mediated proliferation.

Interestingly, all tumors may not inhibit chimeric receptor-mediated proliferation to the same degree, as suggested by the consistently lower inhibitory capacity of supernatant derived from the Renca cell lines compared with supernatants derived from other tumors ($P_2 < 0.01$ compared with any other tumor, Mann–Whitney test, $n = 4$). Therefore, the dual-specific approach may be of greater benefit in those cases where tumor actively inhibits T cell activity. Nevertheless, although proliferation can be mediated by the chimeric receptor, it does not approach the level possible through the endogenous TCR (Figure 4a).

The reason for the inhibition of proliferation by tumor supernatants is not clear at this stage, but may include secretion of immune inhibitory cytokines such as TGF-β from tumor cells. It is unlikely to be due to erbB2 antigen shed from the surface of tumor cells, since similar inhibitory activity was observed using supernatant from antigen-negative tumor cells (Figure 4b).

Although the combination of dual-specific T cells and influenza virus immunization could inhibit tumor growth in mice, tumors were not eradicated. This was despite considerable expansion of T cells as a result of immunization. Intraperitoneal injection of influenza virus results in a nonproductive infection of macrophages leading to effective presentation of viral antigens and a robust immune response, which in this case using SCID mice involved adoptively transferred T cells. Immunization resulted in extensive expansion of T cells systemically including in spleen and peritoneum. Thus, the lack of tumor response was probably not due to insufficient expansion of T cells.

The presence of inhibitory factors within tumor was a potential reason for the low impact on tumor, but T cells were demonstrated to be able to respond against tumor, at least in shorter-term assays *in vitro*, with both specific cytokine secretion and cytolytic activity demonstrated.

Another potential reason for the weak antitumor response included the observed poor tumor localization of T cells. Localization of T cells to tumor was less than 1%, which was much less than that usually associated with a successful response against malignancy or infectious disease. Thus, poor T-cell trafficking was seen as the most likely barrier to overcome to induce effective antitumor responses. Poor trafficking was observed despite administration of influenza virus immunogen to the same anatomical site as tumor. However, tumor tissue is unlikely to be infected with influenza virus, and may lack appropriate inflammatory or danger signals necessary for lymphocyte recruitment. Approaches involving alternate immunogens or vaccination strategies may lead to enhanced trafficking of dual-specific T cells to tumor.

Although this type of treatment did not result in complete regression of tumors in mice, it may still be of value to patients since slower disease progression in patients may afford more opportunity to impact on tumor. Renca tumor grows aggressively in mice even after administration of low numbers of tumor cells, and mice usually fail to survive beyond day 20 if untreated. However, the immunization strategy described here takes almost two weeks before expansion of large numbers of T cells is achieved, by which time tumors have progressed considerably.

Other factors to consider regarding clinical translation of this approach include the number T cells transferred, and *in vitro* culture period of the T cells. One million T cells was chosen as the T cell dose in these experiments as this would correspond to approximately 3×10^9 human T cells when scaled up to patient treatment, which would be a reasonably achievable number of virus-specific T cells to generate from patients. In addition, the T cells used in these experiments were cultured for approximately 2 weeks to achieve sufficient numbers of G418-selected T cells. However, recent reports have demonstrated the superior antitumor activity of shorter-term cultured T cells when used in adoptive immunotherapy for melanoma in mice.[33] It will be of interest in future experiments to determine the relative impact on tumor of various numbers of T cells cultured for different periods.

The nature of the immunogen is a further consideration in the translation of this approach. A live pathogenic virus such as the PR/8 strain of influenza virus may not be appropriate in humans, and inactivated virus similar to existing vaccines may be preferred, although the extent of dual-specific T-cell expansion following this form of immunization compared with live virus is not clear. The feasibility of generating and transducing influenza-specific human T cells has been previously demonstrated.[29] Alternatively, an attenuated virus may be suitable, or a widespread persistent virus such as EBV or CMV may be an effective second specificity to use. The possibility of generating human dual-specific T cells with reactivity against tumor and EBV or CMV has been previously demonstrated *in vitro*.[27,28]

In this study, we attempted to circumvent the poor immunogenicity of tumors by incorporating a second response capability into tumor-specific T cells. Some impact on tumor growth was observed using this approach but the response was suboptimal. Future approaches involving strategies to enhance tumor localization of T cells, and reduce their susceptibility to tumor-derived inhibitory factors, may lead to effective immunotherapies for cancer.

Acknowledgements

This work was supported by grants from The National Health and Medical Research Council of Australia (NHMRC), The Cancer Council of Australia, The Bob Parker Memorial Trust and the Peter MacCallum Cancer Centre Foundation. MK is supported by a Career Development Award from the National Breast Cancer Foundation and NHMRC.

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Dual-specific T cells combine proliferation and antitumor activity

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Published online 4 November 2002; doi:10.1038/nbt756

An effective immune response against cancer requires the activation and expansion of specific T cells. Tumor antigens, however, are generally poor immunogens. To achieve expansion of tumor-reactive T cells *in vivo*, we used a strategy of generating dual-specific T cells that could respond to a powerful immunogen while also possessing tumor reactivity. We generated dual-specific T cells by genetic modification of alloreactive T cells with a chimeric receptor recognizing folate-binding protein, an ovarian cancer–associated antigen. Mouse dual-specific T cells responded *in vitro* to both allogeneic antigen and tumor cells expressing folate-binding protein, and expanded in number *in vivo* in response to immunization with allogeneic cells. Most importantly, the combination of dual-specific T cells and immunization had an antitumor effect *in vivo*. We also generated human dual-specific T cells and characterized the dual-specific nature of individual clones. Assigning the tasks of expansion and tumor reactivity to different receptors within the same lymphocyte may help to overcome the problem of poor immunogenicity of tumor antigens.

Directing the immune system against cancer has long been a goal of immunologists. The many tumor-associated antigens (TAA) with little or no expression in normal tissues are attractive targets that might provide a highly specific antitumor response with little toxicity to normal tissue[1]. In humans, a wide variety of tumor antigens with defined T-cell reactivity are known. Some are currently being assessed as vaccines in clinical trials[2,3], but responses to these vaccines have been infrequent[4]. One reason for this limited success may be the absence of sufficient numbers of tumor-reactive T cells. Indeed, tumor antigens are thought to be poor immunogens.

We and others have investigated the potential of adoptive immunotherapy using large numbers of *in vitro*–activated T cells in an attempt to enhance the number and activity of T cells potentially applicable as therapeutics for a variety of viral and malignant disease[5–8]. In mouse tumor models, adoptive immunotherapy can effectively mediate antitumor effects[9,10]. Humans have also benefited from treatment that includes adoptive transfer of autologous tumor-reactive T cells[7,8], although responses are restricted to only a proportion of patients suffering from one of a limited range of malignancies. This limited success may result from poor persistence of tumor-specific T cells, because large numbers of these activated cultured cells do not survive long *in vivo* after adoptive transfer[11].

With these considerations in mind, we developed a strategy involving the generation of dual-specific T cells that are able to proliferate *in vivo* in response to a powerful immunogen and that also recognize tumor antigen. In this approach, the endogenous T-cell receptor (TCR) provides activation and proliferative signals to T cells in response to the immunogen, whereas tumor specificity is provided by genetic modification with a chimeric receptor that recognizes TAA. The immunogen used in this study was allogeneic major histocompatibility complex (MHC) present on the surface of allogeneic cells. We chose to use allogeneic antigen (alloantigen) because it is well documented and provides a powerful stimulus that induces rapid activation and expansion of alloreactive T cells, which are found in large numbers and have diverse functions and phenotypes in most individuals[12,13]. The chimeric tumor-reactive receptor used was made up of an extracellular domain composed of single-chain antibody specific for the human ovarian TAA folate-binding protein (FBP), and an intracellular signaling domain derived from the γ chain of the human Fc receptor complex, which we have previously shown to induce cytokine secretion and lytic capability in T cells[14].

In this study, we examined the ability of adoptively transferred dual-specific T cells to expand *in vivo* in response to allogeneic immunization and to inhibit tumor growth following immunization with allogeneic cells.

Results

Phenotype and reactivity of alloreactive T cells. In our initial experiments, we characterized the T cells that were generated in a mixed lymphocyte reaction (MLR). Flow cytometric analysis of T cells derived from Thy1.1$^+$ C57BL/6 mice indicated that more than 95% were CD8$^+$ in all experiments. In addition, they had high levels of Thy-1.1$^+$ expression, allowing efficient tracking of alloreactive T cells after adoptive transfer into Thy-1.2$^+$ congenic recipients in subsequent experiments. We analyzed the function of these T cells using a cytokine secretion assay (Fig. 1). T cells raised in the MLR secreted large amounts of interferon-γ (IFN-γ) in response to the allogeneic targets CT26 and BALB/c 3T3 but not the syngeneic targets B16, 24JK, or C57BL/6 splenocytes, thereby demonstrating specific alloreactivity of the T cells. Allogeneic stimulation was necessary for the generation of alloreactive T cells, as T cells raised against a melanocyte differentiation antigen, Trp-2, secreted IFN-γ only in response to peptide-pulsed splenocytes and not in response to allogeneic targets.

Expansion of alloreactive T cells *in vivo*. We next determined whether cultured alloreactive T cells could expand *in vivo* in response to immunization with allogeneic cells. We injected Thy-1.1$^+$

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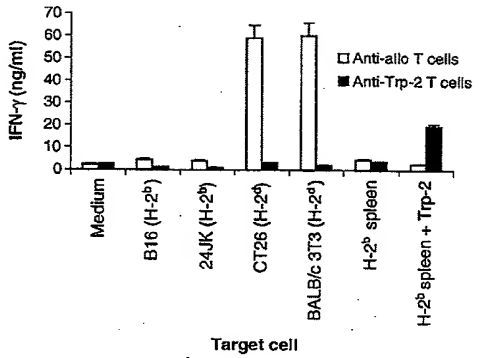

Figure 1. Reactivity of anti-allogeneic mouse T cells. Anti-allogeneic T cells respond to the allogeneic cell lines CT26 and 3T3, but not to the syngeneic lines B16 and 24JK or to syngeneic splenocytes, as demonstrated by IFN-γ release after overnight coculture. The requirement for allogeneic stimulation to generate alloreactive T cells is supported by the absence of alloreactivity of T cells raised against the melanocyte differentiation antigen Trp-2. Results from one representative experiment of three are shown.

alloreactive T cells intravenously into Thy-1.2$^+$ congenic recipient mice, immunized some groups of mice with allogeneic splenocytes, and harvested spleen, lung, and blood 11 days after T-cell transfer. Whereas only small numbers of Thy-1.1$^+$ cells were detected in the absence of immunization (Fig. 2A), a much higher percentage of adoptively transferred T cells was detected following subcutaneous immunization, demonstrating that *in vitro*–cultured, adoptively transferred lymphocytes responded to immunization. The magnitude of expansion in response to immunization was consistently greater than tenfold, with adoptively transferred cells constituting up to 5% of total cells in tissues of immunized mice, thereby constituting a large percentage of CD8$^+$ cells. Surprisingly, intravenous immunization did not result in increased numbers of Thy-1.1$^+$ cells.

The presence of greater numbers of adoptively transferred cells following immunization may have been due to increased survival as well as expansion. The relative contribution of these two effects was not clear. Some expansion of adoptively transferred cells evidently occurred, however, given that the absolute number of Thy-1.1$^+$ cells determined for the tissues harvested, which accounted for only a small fraction of total mouse tissues, was in excess of the 1×10^7 cells transferred. Although subcutaneous immunization seemed to be more effective than intravenous immunization at expanding adoptively transferred T cells, on the basis of results obtained at day 11 after transfer, it seemed possible that this might reflect different kinetics of expansion produced by the different routes of immunization. To investigate this, we immunized mice once on day 2 after transfer and then harvested tissues at several time points (Fig 2B). We again found that subcutaneous immunization was most effective at expanding T cells and that their numbers were highest on day 11 after transfer. Intravenous immunization had virtually no effect at any time point, except for a slight increase in the percentage of Thy-1.1$^+$ cells in the lung on day 6. We therefore used subcutaneous immunization for subsequent experiments. The specificity of immunization required for expansion was demonstrated by the absence of expansion of anti-H-2^d T cells *in vivo* in response to immunization with H-2^k splenocytes (data not shown).

We next considered whether more effective expansion could be achieved by using allogeneic dendritic cells as the immunogen, because dendritic cells are professional antigen-presenting cells (APCs) that express high levels of MHC class I and II molecules. Mice were subjected to adoptive transfer of alloreactive Thy-1.1$^+$ T cells followed by immunization with either allogeneic splenocytes or allogeneic dendritic cells, and tissues were harvested 11 days later. Thy-1.1$^+$ cells constituted about 5% of spleen, lung, and blood following immunization with splenocytes and about 7–10% of these tissues following immunization with dendritic cells (Fig. 2C). However, statistical (Kruskal-Wallis test) analysis of this and a repeat experiment indicated that these percentages were not significantly different. Of interest was the high percentage of adoptively transferred cells in popliteal lymph nodes draining the site of footpad immunization, with up to 39% of lymph node cells being Thy-1.1$^+$. Here also there was a trend for immunization with dendritic cells to induce greater numbers of Thy-1.1$^+$ cells, but the difference was not statistically significant. The percentage of Thy1.1$^+$ cells in lymph node was significantly greater than that in spleen, $P2 < 0.01$ (Kruskal-Wallis test).

To further optimize the expansion of Thy-1.1$^+$ cells *in vivo*, we investigated the effect of immunizations on different days after transfer and with varying numbers of allogeneic splenocytes. The greatest increase in Thy-1.1$^+$ cells in the spleen was seen following subcutaneous immunization on day 1, 2, or 3 after adoptive transfer (Fig. 3A), and these increases were approximately equivalent. Immunization on day 0 resulted in only slight increases in the percentage of Thy-1.1$^+$ cells. Although the absolute percentage of adoptively transferred cells was lower than in the previous experiments—reflecting the use of only one immunization instead of three—up to sixfold expansion of Thy-1.1$^+$ cells occurred with immunization as compared to no immunization.

We next considered whether varying the immunizing dose could affect the expansion of adoptively transferred T cells. Mice were immunized with varying numbers of allogeneic splenocytes on day 2 after transfer of Thy-1.1$^+$ cells, and spleens were harvested on day 11. Immunization with 5×10^7 allogeneic splenocytes produced the largest expansion of Thy-1.1$^+$ cells, approximately tenfold greater than in non-immunized mice (Fig. 3B). A lesser increase in Thy-1.1$^+$ cells (approximately threefold) occurred in mice receiving either 5×10^6 or 2×10^8 allogeneic splenocytes. The lowest immunizing dose, 5×10^5 cells, was ineffective at increasing the percentage of adoptively transferred cells. These results were confirmed in a second experiment. We therefore used an immunizing dose of 5×10^7 splenocytes starting on day 2 in subsequent experiments.

Phenotype and activity of dual-specific T cells. To test the application of this immunization strategy to tumor-reactive lymphocytes, we generated dual-specific T cells by genetic modification of alloreactive T cells with a receptor, MOv-γ, recognizing the ovarian cancer–associated antigen FBP. Initially, we wished to determine the transgene expression and reactivity of dual-specific cells against both allogeneic cells and FBP. We confirmed that the dual-specific cells expressed the chimeric MOv-γ receptor by flow cytometric analysis of the dual-specific T cells following staining with anti-idiotype antibody (Fig. 4A).

The activity and specificity of T cells generated by genetic modification of T cells from an MLR was determined by a cytokine release assay after overnight coculture with target cells. Alloreactivity of T cells transduced with either MOv-γ or green fluorescent protein (GFP; control) was evident from the high levels of IFN-γ secreted in response to the allogeneic target CT26 but not the syngeneic targets MC38 and 24JK (Fig. 4B). FBP reactivity of MOv-γ-transduced T cells was demonstrated by their secretion of IFN-γ in response to the FBP$^+$ cell lines 24JK-FBP and IGROV but not the FBP$^-$ cell lines 24JK and 888. The requirement for MOv-γ expression was confirmed by the observation that GFP-transduced T cells did not secrete IFN-γ in response to FBP$^+$ targets.

Before proceeding with *in vivo* tumor studies, it was important to first determine whether dual-specific T cells could expand *in vivo* in response to allogeneic immunization, as occurred with alloantigen-reactive Thy-1.1$^+$ T cells (Fig. 2A). We treated mice with 1×10^7 Thy-1.1$^+$ dual-specific T cells on day 0 followed by subcutaneous immunization with 5×10^7 allogeneic splenocytes on days 2, 5, and 8. Spleens were har-

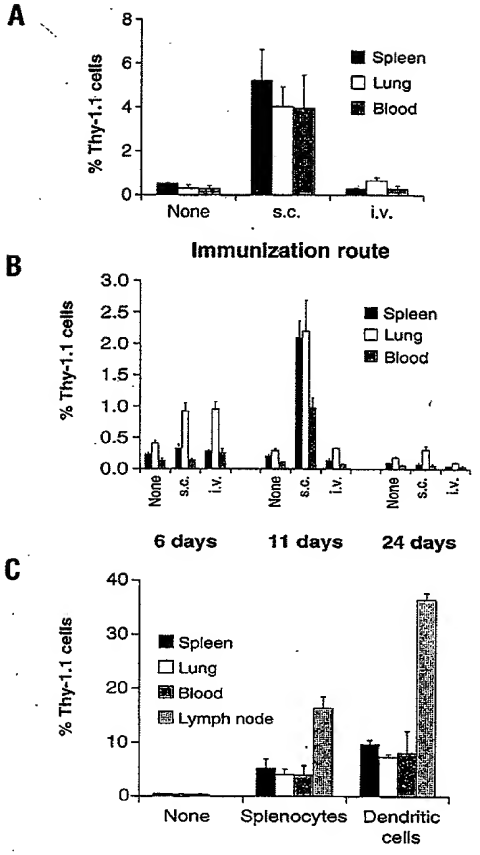

Figure 2. *In vivo* expansion of adoptively transferred alloreactive T cells. 1×10^7 H-2^d-reactive Thy-1.1$^+$ T cells were adoptively transferred into Thy-1.2$^+$ recipients. Some mice received immunization with 5×10^7 H-2^d splenocytes two, five, and eight days later (A) or only two days later (B). (A) Small numbers of Thy-1.1$^+$ cells were present in spleen, lung and blood of nonimmunized mice harvested on day 11 after transfer. Mice that received subcutaneous immunization had over tenfold more Thy-1.1$^+$ cells in tissues than nonimmunized mice. In contrast, adoptively transferred cells did not expand in mice that received intravenous immunization. (B) When tissues were harvested at different time points after adoptive transfer and a single immunization two days later, maximal expansion of transferred cells occurred on day 11, and only with subcutaneous immunization. (C) A comparison of splenocytes with dendritic cells as immunogens revealed these two conditions to be approximately equivalent in their ability to induce expansion of transferred cells *in vivo*. Thy-1.1$^+$ cells comprised a large percentage of cells in lymph nodes draining the immunization site. Results from one representative experiment of three (A) or two (B, C) are shown, presented as the mean percentage ± s.e.m. with three mice per group.

vested on day 11 and analyzed for the percentage of Thy-1.1$^+$ cells. A more than tenfold increase in the percentage of Thy-1.1$^+$ cells as a result of immunization was consistently observed (Fig. 4C).

Antitumor effect of dual-specific T cells. To determine whether dual-specific T cells in combination with allogeneic immunization were effective at protecting mice from tumor challenge, we injected mice with dual-specific T cells, immunized some mice with allogeneic splenocytes on day 2, and then challenged some mice from each category with tumor on day 9. The majority of mice that received dual-specific T cells and immunization were effectively protected from a challenge dose of 2×10^4 24JK-FBP tumor cells (Fig. 5A) for the entire observation period of more than 40 days. Mice treated with immunization alone, or injection of dual-specific T cells alone, were not protected against tumor challenge as compared to untreated mice, demonstrating that both allogeneic immunization and dual-specific T-cell transfer are required for optimal antitumor effect.

To determine whether the combination of dual-specific T cells and immunization was effective at treating tumor cells injected at a later time, mice were injected with 2×10^5 tumor cells, and adoptive transfer of T cells was done one day later. Immunization with allogeneic splenocytes was then performed on days 3, 6, and 9 after the initial tumor injection. Thus a key element of treatment (immunization) was not applied until tumors were three days old and easily palpable. In three separate experiments, tumor growth in mice receiving dual-specific T cells and immunization was significantly less in these mice than in untreated mice and mice receiving dual-specific T cells alone (Fig. 5B). Treatment with nonspecific T cells and immunization had no effect, as did treatment of FBP-negative tumor cells with dual-specific cells and immunization. The requirement for both FBP antigen on tumor cells and MOv-γ expression on T cells, along with immunization, in order for tumor inhibition to occur was thus clearly demonstrated.

Characterization of human dual-specific T cells. To assess the feasibility of this approach for treating human cancers, we attempted to generate human dual-specific T cells by MLR and gene modification with the MOv-γ receptor. Functionally, the bulk MOv-γ-transduced T-cell population was demonstrated to be both alloreactive and FBP reactive (Fig. 6A), whereas nontransduced T cells were not FBP reactive. To determine whether individual T cells within the bulk population were dual specific, we performed cloning by limiting dilution and examined a total of 140 clones in three experiments for reactivity towards allogeneic targets and FBP$^+$ targets. Of these clones, 52% were alloreactive and 68% of these were FBP reactive. Thus, 35% of the bulk T-cell population was dual specific. The activity of five representative clones demonstrating dual specificity is evident from the results of a GM-CSF release assay (Fig. 6B). GM-CSF was chosen as the indicator of reactivity because it is secreted by both type 1 and 2 T cells, in contrast to IFN-γ, which is secreted predominantly by type 1 T cells. Notably, the bulk T-cell population was made up largely of CD4$^+$ T cells (83%), with smaller numbers of CD8$^+$ cells (13%) and some CD4$^+$CD8$^+$ (4%) and CD4$^-$CD8$^-$ (1%) cells also present. Of the five representative clones presented here, clones 1, 2, and 3 were CD4$^+$, clone 4 was CD4$^+$CD8$^+$, and clone 5 was CD8$^+$.

Discussion

The potential effectiveness of immunization with TAA as a therapy for cancer is clearly evident from results in mouse tumor models with immunogenic tumors expressing nonself antigens[15]. However, effective immunization against tumor-associated self antigens is difficult[16]. In humans, too, despite limited success, cancer therapy using TAA has been ineffective[4]. Apparently, most tumor antigens, as currently used, are relatively weak stimulators of immunity. The limited effectiveness of tumor vaccines and other immunotherapeutic strategies for cancer treatment may be partly a consequence of the inability to generate large numbers of activated, high-avidity antitumor T cells.

To address this problem, we have described the generation, by genetic modification of alloreactive cells, of dual-specific T cells that can expand *in vivo* in response to a strong immunogenic stimulus (alloantigen) while retaining antitumor reactivity. Alternative specificities for the immunogen component of the dual-specific system are also possible, including viral proteins or even live virus, which would be expected to initiate a vigorous response *in vivo*. Indeed, a recent report[17] has described the generation of human T cells that are reactive with GD2 neuroblastoma antigen and Epstein-Barr virus (EBV) *in vitro*. The authors propose that the prevalence in the human population of EBV in latent form would provide both a ready source of EBV-specific T cells for conversion to dual specificity, and a convenient immunogen that could lead to enhanced survival of adoptively transferred dual-specific T cells. Viral antigens and alloantigens are both potent

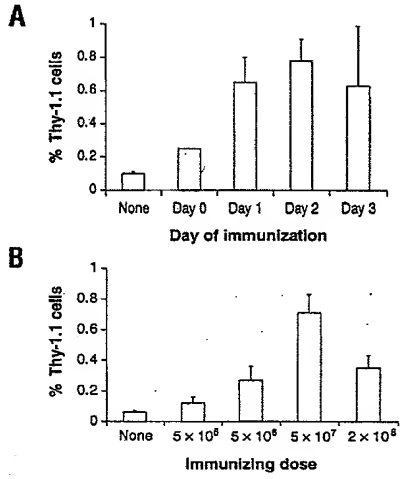

Figure 3. Timing and dose of immunization. (A) Mice received 1×10^7 alloreactive Thy-1.1$^+$ T cells followed by a single subcutaneous immunization with 5×10^7 allogeneic splenocytes on either day 0, 1, 2, or 3 after adoptive transfer, or no immunization. The percentage of Thy-1.1$^+$ cells, determined in spleens harvested 11 days after adoptive transfer, was roughly equal in mice immunized on day 1, 2, or 3 and was comparatively lower in mice immunized on day 0 or not immunized. (B) Mice received 1×10^7 alloreactive Thy-1.1$^+$ T cells followed two days later by subcutaneous immunization with various numbers of allogeneic splenocytes. Some expansion of Thy-1.1$^+$ cells was seen after 5×10^6 splenocytes, but optimal expansion occurred with a dose of 5×10^7 splenocytes. In both (A) and (B), results are presented as the mean percentage ± s.e.m. from two experiments with three mice per group in each experiment.

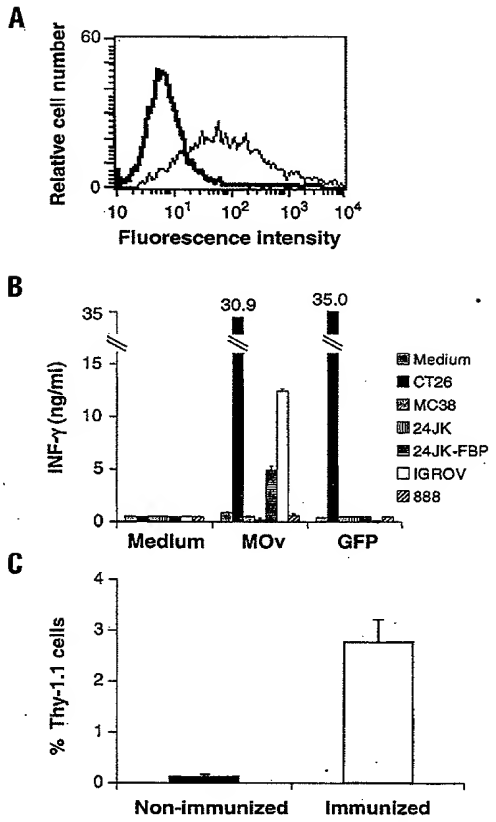

Figure 4. Phenotype, activity, and expansion of dual-specific T cells. (A) Mov-γ expression. Dual-specific T cells expressed MOv-γ, as demonstrated by flow cytometry after staining with anti-idiotype antibody (thin line) compared to isotype control (thick line). Results are representative of many experiments. (B) Dual-specific activity. T cells were cocultured with the target cells listed, and supernatants were assayed by ELISA for the presence of IFN-γ. Anti-allogeneic activity of both MOv-γ- and GFP-transduced T cells was demonstrated by IFN-γ secretion in response to the allogeneic target CT26 (H-2^d) but not the syngeneic targets MC38 and 24JK (H-2^b). Anti-FBP reactivity of the MOv-γ-transduced T cells was evident from their secretion of IFN-γ in response to 24JK-FBP but not 24JK cells, and in response to the human FBP$^+$ ovarian cancer cell line IGROV but not the FBP$^-$ human melanoma cell line 888. T cells did not secrete IFN-γ in the absence of target cells. Results of one representative experiment of five are shown. (C) Expansion of dual-specific T cells. Spleens of mice receiving 1×10^7 dual-specific T cells followed by immunization were analyzed for Thy-1.1$^+$ cells 11 days after transfer. A more than tenfold greater percentage of Thy-1.1$^+$ cells was consistently observed after immunization as compared to no immunization. Results are mean ± s.e.m. of three experiments with three mice per group.

stimulators of immunity, and both that study and ours support the potential of the dual-specific T-cell strategy for the improvement of T-cell immunotherapy. Importantly, the current study supports the feasibility of the approach against tumors *in vivo* in an animal model.

We examined several parameters important in the expansion of dual-specific cells *in vivo* and, unexpectedly, found that intravenous immunization did not induce expansion of adoptively transferred cells. The reason for this is not clear, although it is possible that subcutaneously delivered allogeneic cells gained access to the same compartment as adoptively transferred T cells via the lymphatic system, whereas allogeneic cells delivered intravenously were cleared more rapidly. Intravenous delivery of allogeneic cells has been demonstrated to reduce subsequent responses to alloantigen in other systems[18].

The most effective immunization procedure among those tested was the subcutaneous injection of 5×10^7 allogeneic splenocytes in the mouse footpads and flank on days 2, 5, and 8 after adoptive transfer. Both alloreactive T cells in initial experiments and dual-specific T cells in subsequent experiments expanded in response to immunization to constitute from 3–10% of total cells in spleen, lung, and blood. Although we did not restrict our calculations to express the numbers of adoptively transferred cells as a percentage of the total CD8$^+$ cells in the tissues, it is likely that in the spleen, for example, Thy-1.1$^+$ cells make up a large percentage of CD8$^+$ cells.

Our result demonstrating that *in vitro*–activated and cultured T cells can expand *in vivo* following immunization is notable in itself, as *in vitro* culture may afford the opportunity for manipulation of T cells to achieve optimal antitumor potential, and yet few studies have used cultured cells in this manner. Interestingly, very high percentages of cells in lymph nodes draining immunization sites consisted of adoptively transferred Thy-1.1$^+$ cells. This would suggest that, although cultured activated T cells are often thought to have an effector phenotype poorly suited for normal trafficking and function *in vivo*, there seems to be at least a population of cells present that are able to access normal secondary lymphoid tissue and respond to antigen by proliferating.

The tumor specificity here is provided by genetic modification with a chimeric receptor incorporating a single-chain antitumor antibody against ovarian cancer. In previous work from our laboratory, we have described antitumor effects of adoptively transferred mouse T cells transduced with chimeric receptor[14]. However, the tumors treated in those studies were intraperitoneal xenografts of human ovarian cancer cells or lung metastases of a syngeneic sarcoma expressing FBP. The current study concerns the treatment of subcutaneous tumor, a different disease that is inherently more difficult to treat using adoptively transferred T cells. These considerations—along with our data showing that transduced T cells alone had little or no effect in the prevention or treatment of subcutaneous tumor, whereas transduced T cells and

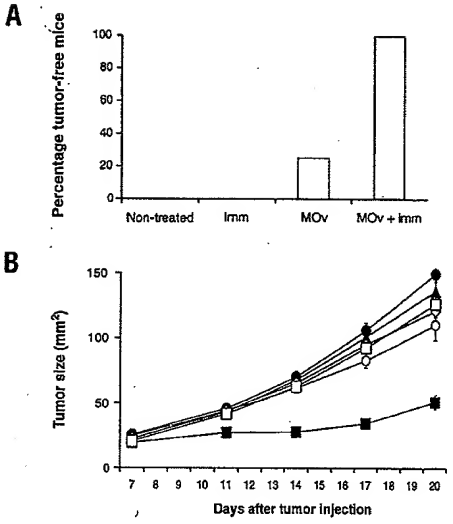

Figure 5. The combination of adoptive transfer of dual-specific T cells and immunization inhibits tumor growth. (A) Tumor prevention with dual-specific T cells. Mice received either no treatment or MOv-γ-transduced (dual-specific) T cells (1×10^7); some groups also received subcutaneous immunization with 5×10^7 allogeneic splenocytes two days later. Mice were challenged with tumor seven days after immunization. Tumors grew progressively in untreated mice and in those receiving immunization alone. The majority of mice receiving dual-specific T cells alone were not protected from tumor challenge, but all mice receiving a combination of dual-specific T cells and immunization were protected from challenge. Dual-specific T cells and immunization was more effective than any other treatment (Kruskal-Wallis test, $P2 < 0.05$). (B) Tumor treatment with dual-specific T cells. Mice treated with subcutaneous injection of tumor cells, followed by T cells one day later and immunization beginning three days after tumor-cell injection. 24JK-FBP tumors grew progressively in mice receiving either no treatment (filled triangles), GFP-transduced T cells with immunization (filled circles), or dual-specific (MOv) T cells alone (open circles). 24JK-FBP tumor growth was significantly inhibited by the combination of dual-specific T cells and immunization (filled squares). The requirement for FBP expression in tumors for this effect was evident from the absence of 24JK (antigen-negative) tumor growth inhibition by dual-specific T cells and immunization (open squares) compared to untreated 24JK tumor (open diamonds). In both (A) and (B), results from one representative experiment of three are shown, presented as the mean ± s.e.m. with five mice per group.

immunization had considerable effect (Fig. 5)—serve to emphasize the extent of the improvement in antitumor efficacy offered by the dual-specific approach. The wider applicability of this approach to other malignancies is evident from work done in our laboratory and others to develop chimeric receptors with specificity for a range of antigens, including those overexpressed on cancers of the colon[19,20], breast[21], and kidney[22] and on tumor vasculature[23].

Other possible ways to generate dual-specific T cells include genetic modification of alloreactive cells with α and β chains of antitumor TCR[24], and modification of tumor-specific T cells with alloreactive TCR that may then be able to expand in response to immunogen. In this latter approach, knowledge of the antigen responsible for tumor specificity is not necessary. Alternatively, because many T cells cross-react with alloantigen in addition to their primary TCR specificity[25], it may be possible to detect antitumor T cells that also respond naturally to allogeneic antigen. This approach might require screening a large panel of allogeneic MHC types to determine the optimal stimulator MHC, but has the advantage of not requiring genetic modification of T cells.

Because alloantigen produces particularly potent activation and expansion of T cells with other inherent specificities, it is worth considering the possible implications of this approach for the induction of autoimmunity in recipients of therapy. The transfer of large numbers of alloreactive T cells, and the further generation of alloreactive T cells *in vivo* after immunization, could conceivably lead to the activation of T cells cross-reactive with autoantigens. Of particular concern might be the activation of autoreactive $CD4^+$ T cells that could then induce the production of autoreactive antibodies from B cells. Although there is no direct evidence that this might occur, the possibility is worth bearing in mind.

The dual-specific T cells used in this study were reactive against two nonself antigens (human FBP and alloantigen), and it is therefore not clear whether this approach could be duplicated for the majority of tumor-associated antigens, which are self antigens. However, studies by other investigators of T-cell ontogeny in double TCR-transgenic mice have demonstrated the ability of the presence of a non-self-reactive TCR to rescue T cells also bearing a self-reactive TCR from central deletion[26]. Nevertheless, although there is no effective endogenous immune response to FBP, as evidenced by the aggressive growth of FBP-expressing sarcoma at a rate equivalent to the parental FBP-negative cell line, it is still possible that other FBP-induced elements of the immune system, not potent in themselves, contribute to the observed effect.

Also worth considering is that because no effective endogenous immune response is raised against this tumor, 24JK-FBP can be considered a poorly immunogenic tumor in this model. Indeed, the adoptively transferred T cells detect antigen in an MHC-unrestricted manner, and will not expand in response to tumor antigen in a traditional APC-mediated fashion.

It is also difficult to assess the extent of autoimmunity that may be induced in a self-antigen setting where low-level expression of the target TAA may occur on normal tissue. Unfortunately, single-chain antibodies specific for mouse cell-surface TAA are rare, and no chimeric receptors incorporating them are currently available, so it is impossible to answer this question at present. A model system involving a chimeric receptor targeting rat erbB-2 in mice transgenic for rat erbB-2 is under development in our laboratory and may help provide answers to these questions.

Another question concerns the possible contribution of T cells reactive to FCS, which was used in the culture of both tumor cells and T cells. Although some FCS-specific T cells could conceivably be generated in culture, we would expect their numbers to be very low in comparison to those of the alloreactive T cells generated in response to the powerful alloantigen stimulus. Indeed, dual-specific T cells against MHC class I–positive target cells that have been exposed to FCS, but do not express FBP, show very little activity as measured by IFN-γ release (Figs. 1 and 4B). It is unlikely that FCS reactivity contributes substantially to *in vivo* T-cell expansion, as FCS was not included in our original immunization regimen. The lack of an effect of T cells against FBP-negative tumors in mice (Fig. 5B) was also an indication that anti-FCS activity does not contribute substantially to antitumor effects. Adding FCS to the immunization schedule did not result in a further increase in the number of adoptively transferred T cells (data not shown). Thus it is not likely that FCS contributes markedly to the observed antitumor effects, and it would not need to be included as an immunogen if this strategy were applied in humans.

We have focused on the effect of immunization on the numbers of dual-specific T cells *in vivo*, and it is likely that the increase in these cells contributes to the observed antitumor effect. It is also possible, however, that immunization activates dual-specific T cells that could enhance the antitumor activity of dual-specific T cells. Indeed, activation in this manner might overcome the induction of tolerance to tumor antigen or circumvent active suppression[27] of tumor-reactive cells.

It is also worth considering that the antitumor effects of dual-specific T cells may be augmented by help from endogenous recipient cells involved in an immune response to alloantigen. In addition, it is

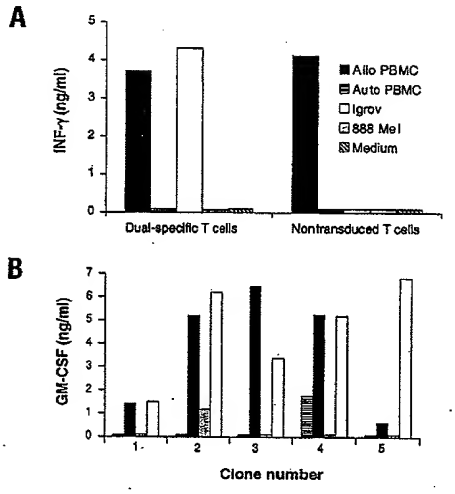

Figure 6. Reactivity of human dual-specific T cells. (A) Human T cells generated against allogeneic antigen and transduced with MOv-γ-secreted IFN-γ in response to both allogeneic PBMC and the FBP+ ovarian cancer cell line IGROV and not in response to autologous PBMC, FBP- 888 melanoma cells, or medium alone, thereby demonstrating the dual specificity of the bulk T-cell population. The absence of IFN-γ release by nontransduced T cells incubated with IGROV demonstrates that MOv-γ transduction is required for reactivity against FBP. (B) 35% of transduced T-cell clones from the bulk transduced T-cell population reacted against both allogeneic cells and FBP+ target cells. Five representative T-cell clones are shown that secreted GM-CSF in response to both allogeneic PBMC and IGROV, thereby demonstrating that individual T cells can be dual-specific. Smaller amounts of GM-CSF were secreted in response to autologous PBMC and the 888 melanoma cell line. Nontransduced clones did not secrete GM-CSF in response to FBP+ cells (data not shown). Generation, characterization, and cloning of human dual-specific T cells was done three times with similar results.

possible that adoptively transferred mono-specific T cells (alloreactive only) present in the transferred cells contribute indirectly to enhancing the antitumor activity of chimeric receptor–expressing T cells following immunization. The relative contribution from these indirect effects is not clear from this study, although there is probably a significant mechanistic contribution resulting from the large increase in the numbers of dual-specific cells following immunization.

The adoptively transferred bulk mouse T-cell population is probably composed of a mixture of cells specific for alloantigen alone or FBP alone in addition to dual-specific T cells. The relative increase in these separate populations *in vivo* following immunization is not known. It is likely, however, that dual-specific T cells are present *in vivo* for at least 11 days, because T cells re-isolated from spleens and lymph nodes of recipient mice at this time point were able to proliferate in response to re-stimulation with allogeneic splenocytes *in vitro* in the presence of the neomycin analog G418, which selected for transduced T cells, and to retain chimeric MOv-γ expression and dual reactivity (data not shown).

It is not clear from this study what contribution different T-cell subsets make to the observed effect in mice, although it is likely that $CD8^+$ T cells contribute substantially because these represent a large percentage (>95%) of the mouse T cells used for adoptive transfer. Investigators have consistently found a preponderance of $CD8^+$ T cells following stimulation of mouse splenocytes by MLR or lectin, although the reason for this polarization is not clear. In humans, by contrast, MLR results in mainly (often >80%) $CD4^+$ cells in some cases. Although $CD4^+$ may contribute to antitumor reactivity, in applying this approach clinically it might be advantageous to include strategies that involve enrichment of $CD8^+$ cells or selection of an immunogen that preferentially expands $CD8^+$ cells.

The strategy described here requires culturing of T cells to enrich for alloreactive cells. Activation in culture is also necessary to achieve integration of retroviral vectors in their current form. This can potentially decrease the function of T cells *in vivo* following adoptive transfer as compared to noncultured T cells. Earlier investigators have observed other examples of decreased function, whereby transfer of cultured allogeneic T cells reduced their capacity to induce graft-versus-host disease in allogeneic recipient animals[28,29]. In our approach, large numbers of adoptively transferred T cells persist (and probably expand), and *in vitro* reactivity is high; however, improvements could perhaps be achieved by modifying the culture or transduction conditions or by minimizing *in vitro* culture. To this end, an amended strategy involving isolation of naive T cells using appropriate tetramers and minimal culture with retroviral or lentiviral vectors might be fruitful.

In conclusion, many hurdles must be overcome in order to achieve effective immunization against tumor antigens, not the least of which is the need to effectively circumvent self-tolerance to TAAs that are generally expressed in other, normal tissues. By delegating the responsibility for expansion and activation to a highly responsive yet separate receptor from that encoding tumor reactivity, it may be possible to overcome the problem of weak tumor immunogenicity. The production of dual-specific T cells provides an alternative strategy for vaccine development and immunization because it offers a means to generate a potent reaction against poorly immunogenic antigens.

Experimental protocol

Cell lines and mice. 24JK is a methylcholanthrene-induced sarcoma of C57BL/6 mice, and 24JK-FBP was derived from this line by transduction with cDNA encoding human FBP. B16 is a melanoma and MC38 is a colon adenocarcinoma of C57BL/6. CT26 is a colon carcinoma and BALB/c 3T3 is a fibroblast line of BALB/c mice. IGROV is a FBP+ human ovarian cancer cell line, and 888 is a FBP- human melanoma cell line. The retroviral producer cell lines used were GP+E86 for mouse studies and PG13 for experiments with human cells. The retroviral construct encoding the chimeric receptor, MOv-γ, was composed of extracellular single-chain MOv18 anti–ovarian carcinoma antibody specific for human FBP[30,31] linked to the transmembrane and cytoplasmic domains of the human Fc receptor γ chain. This chimeric receptor was cloned into the vector pSAMEN[32], where expression was driven by the long terminal repeat promoter region of the Moloney murine leukemia virus. A neomycin-resistance gene (neomycin phosphotransferase) is included in this vector under the transcriptional control of an internal ribosomal entry site. Previous studies characterizing the parental MOv18 antibody[31] indicate that it has an affinity constant of $2 \times 10^8\ M^{-1}$ and that the number of antigenic determinants on human ovarian cancer cells is on the order of 1×10^7.

Producer cell lines were generated by genetic modification with the pSAMEN vector encoding either the chimeric receptor MOv-γ or GFP, using a method similar to that previously reported[20], followed by transduction of GP+E86 and PG13. All cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 5 μg/ml gentamycin, and 5×10^{-5} M 2-mercaptoethanol. Mice used in these experiments were C57BL/6 and BALB/c produced at the Animal Production Area, National Cancer Institute (Frederick, MD). Thy-1.1 C57BL/6 mice (B6.PL-Thy1^a/Cy) were obtained from Jackson Laboratories (Bar Harbor, ME). The experimental design was approved by the Animal Experimental Ethics Committee, National Insitutes of Health.

Antibodies and flow cytometry. Antibodies (1 μg) specific for CD4, CD8, H-2K^b, AND Thy-1.1, and rat IgG isotype controls (Pharmingen, San Diego, CA), were incubated with 1×10^6 cells. The cells were then washed in PBS and assayed by flow cytometry. For the detection of chimeric MOv-γ receptor, cells were incubated with a phycoerythrin-conjugated antibody directed at the idiotype of the single-chain antibody making up the extracellular portion of the receptor.

Generation of alloreactive and dual-specific T cells. Mouse alloreactive T cells were generated by MLR involving coculture of 2×10^6 C57BL/6 Thy-1.1$^+$ splenocytes with 2×10^6 irradiated (2,000 Rad) BALB/c splenocytes per well in 24-well plates. Human interleukin-2 (IL-2; 60 IU/ml) (Chiron Corp, Emeryville, CA) was added on day 2 and every second day thereafter. Cultures were maintained

at a cell density of 0.5×10^6–3×10^6 cells per well. Cells were restimulated every 7 days by seeding at 5×10^5 T cells per well and adding 2×10^6 irradiated BALB/c splenocytes and IL-2. T cells were used for adoptive transfer on day 5 or 6 after one or two re-stimulations. Dual-specific mouse T cells were generated by MLR in similar conditions, except that 1×10^5 non-irradiated GP+E86 retroviral packaging cells were preseeded in 1 ml into each well the night before the MLR. Subsequent re-stimulations of transduced mouse T cells were done in the presence of the neomycin analog G418 (500 μg/ml). Dual-specific human T cells were generated in MLR similarly to mouse dual-specific cells, except that irradiated allogeneic PBMC were used as stimulators and retroviral supernatant from PG13 producer lines was used as described[20] for two transductions.

Determining percentage of Thy-1.1$^+$ cells in tissues. Spleens and popliteal lymph nodes were harvested and crushed; lung and tumor were digested with hyaluronidase (100 μg/ml), collagenase (1 mg/ml), and DNase (30 U/ml) (all from Worthington Biochemical, Lakewood, NJ) for six hours. 5×10^6 cells were stained with phycoerythrin-conjugated anti-Thy-1.1 and analyzed by flow cytometry. The amount of Thy-1.1$^+$ cells present in each tissue was determined as the percentage of total cells in that tissue.

Adoptive transfer of T cells and immunization. For tumor-prevention studies, mouse T cells were harvested from 24-well plates, washed twice in PBS, and resuspended at 2.5×10^7 cells/ml, and 0.4 ml were injected intravenously via the tail vein into mice. For tumor treatment studies, 1×10^6 T cells (or 3×10^6 T cells in some experiments) in 0.4 ml of PBS were injected intravenously. Unless otherwise stated, immunization of mice was done using 5×10^7 allogeneic splenocytes in 0.2 ml PBS, either intravenously (tail vein) or subcutaneously (0.05 ml footpads and 0.1 ml flank) on days 2, 5, and 8 after adoptive transfer. In some experiments, 1×10^7 allogeneic dendritic cells were used as immunogen. Dendritic cells were generated from bone marrow isolated from the legs of BALB/c mice and cultured in murine IL-4 and GM-CSF, as reported previously[33].

Cytokine release assays. T-cell reactivity against alloantigen and FBP was determined by co-incubating 1×10^5 T cells with 1×10^5 target cells in 96-well plates overnight and then assaying the supernatants for IFN-γ or GM-CSF by enzyme-linked immunosorbent assay (ELISA) using commercially available reagents according to the manufacturers instructions (Endogen, Woburn, MA).

Tumor studies. For tumor-prevention studies, mice were injected with 1×10^7 T cells on day 0 and immunized by subcutaneous injection with 5×10^7 non-irradiated allogeneic splenocytes on day 2. Mice were challenged subcutaneously with either 24JK or 24JK-FBP cells on day 9, and tumor growth was monitored. For tumor-treatment studies, mice were irradiated with a sublethal dose of radiation (600 cGy) to delay the onset of an endogenous antibody response to human FBP that would interfere with the interaction between FBP on tumor and anti-FBP receptor on dual-specific T cells. On the same day, mice were injected subcutaneously with 2×10^5 tumor cells. Mice received adoptive transfer of T cells one day later. Immunization with 5×10^7 irradiated (2000 cGy) allogeneic splenocytes was performed on days 3, 6, and 9 after tumor injection.

Competing interests statement

The authors declare that they have no competing financial interests.

Received 10 April 2002; accepted 19 September 2002

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